1-1972

Form and Level of Coumarin in Deer's Tongue, *Trilisa odoratissima*

Francis A. Haskins
University of Nebraska-Lincoln, fhaskins@neb.rr.com

H. J. Gorz
University of Nebraska-Lincoln

R. C. Leffel
University of Nebraska-Lincoln

Published in *Economic Botany* (January-March 1972) 26(1): 44-48.

Form and Level of Coumarin in Deer's Tongue, *Trilisa odoratissima*[1]

F. A. Haskins, H. J. Gorz and R. C. Leffel[2]

[1] Contribution from the Plant Science Research Division, Agricultural Research Service, United States Department of Agriculture, and the Nebraska Agricultural Experiment Station, Lincoln, Nebraska. Published with the approval of the Director as Paper No. 3055, Journal Series, Nebraska Agricultural Experiment Station. The work reported was conducted under Nebraska Agricultural Experiment Station Project No. 12-50, and was supported in part by the National Science Foundation (Grant No. GB-8280).

[2] Bert Rodgers Professor of Agronomy, University of Nebraska; and Research Geneticist and Research Agronomist, Plant Science Research Division, Agricultural Research Service, United States Department of Agriculture, Lincoln, Nebraska and Beltsville, Maryland, respectively.

Submitted for publication March 8, 1971.

Fresh leaves of deer's tongue contain large quantities (more than 10% of the dry weight, in some cases) of *o*-hydroxycinnamic acid (*o*-HCA). Both *cis*- and *trans*-*o*-HCA are present, and both isomers exist in the fresh tissue predominantly as glucosides. Cured deer's tongue leaves contain relatively high levels of coumarin and lower amounts of *o*-HCA glucosides. It is probable that during the curing process *cis*-*o*-HCA glucoside is hydrolyzed by an endogenous β-glucosidase, and that the liberated *cis*-*o*-HCA lactonizes spontaneously to form coumarin.

Leaves of deer's tongue, *Trilisa odoratissima* (J. F. Gmel.) Cass., a coumarin-containing plant indigenous to wooded areas in southeastern United States, are used in the tobacco industry, particularly in cigarette mixtures (1, 9). The coumarin contributed by the leaves is said to enhance existing flavors and to "fix" the natural taste of the tobacco (1).

Coumarin is the lactone of *cis*-*o*-hydroxycinnamic acid (*cis*-*o*-HCA). Bound coumarin in sweetclover (*Melilotus alba* Desr.) has been identified as the β-D-glucoside of *cis*-o-HCA (7), and the immediate precursor of this glucoside is the β-D-glucoside of *trans*-*o*-HCA (8). Previous work has shown that in normal, healthy sweet clover leaves, essentially all of the coumarin is present in the bound form (3). Extensive conversion of bound coumarin to the free form occurs upon disruption of the sweetclover leaf tissue. This conversion is effected by a specific β-glucosidase which hydrolyzes *cis*-*o*-HCA glucoside, liberating *cis*-o-HCA which lactonizes spontaneously to yield coumarin (3, 8). In leaves of sweet vernal grass (*Anthoxanthum odoratum* L.) and sweet grass [*Hierochloe odorata* (L.) Beauv.] (3), as well as tonka bean (*Dipteryx odorata* Willd.) (4) and various species of *Trigonella* (2), the relationship between free and bound coumarin appears to be very similar to that observed in sweetclover.

A search of the literature failed to reveal information on the amount of coumarin present or on the form in which the compound exists naturally in the deer's tongue plant. Therefore, the work reported here was undertaken.

Materials and Methods

Samples of cured whole leaves, pulverized leaf tissue, and seed of deer's tongue were obtained through the kind assistance of Dr. William T. Fike, Crop Science Department, North Carolina State University. Five of the cured whole leaves were individually weighed (air-dried weights ranged from 541

to 972 mg), and each was extracted by immersion in 50 ml of boiling water in a 125-ml Erlenmeyer flask, followed by autoclaving at 120° for 30 minutes. Five other cured leaves were separated into midrib and blade portions. Portions were separately weighed (weight ranges: midrib – 99 to 231 mg, blade – 490 to 825 mg) and each was extracted as indicated for whole leaves. Each of five samples (weights from 47.9 to 53.8 mg) of the pulverized leaf tissue was immersed in 10 ml of water in a 20 x 150 mm test tube preheated in a boiling water bath, and the suspensions were autoclaved for 30 minutes. A single sample of 10 seeds (weight 4.5 mg) was extracted with 10 ml of water as indicated in the preceding sentence. Samples of all extracts were decanted into clean test tubes and were held in a freezer for later assay. Percentage of oven-dry material (dried overnight at approximately 100°) was determined for each type of sample to permit expression of o-HCA content on a dry weight basis.

To obtain fresh leaf tissue for assay, several plantings of deer's tongue seed were made in growth chambers held at approximately 27° and lighted with cool white fluorescent tubes. Germination was poor, and growth of the seedlings was extremely slow. Eventually a few plants were obtained, and approximately 3 months after planting, one young leaf from each of three plants was harvested for *o*-HCA and dry matter determinations. Subsequent to this initial sampling, plants were repotted and were moved from the growth chamber to the greenhouse. Growth of the plants in the greenhouse was considerably more rapid than initial growth in the chamber. Approximately 2 ½ months after plants had been moved to the greenhouse, the youngest leaf greater than 2.5 cm in length and the longest leaf were harvested from each of nine plants. Midribs were ex*cis*ed from the longest leaves, and the midribs were then split longitudinally. Half of each midrib was used for dry matter determination, and half was extracted for *o*-HCA assay. Similarly, half of the remaining leaf blade was used for dry matter determination, and half for *o*-HCA assay. Midribs were not ex*cis*ed from the youngest leaves. Rather, the leaves were cut into two halves at the midrib; half of each was used for dry matter determination and half for *o*-HCA assay. Extracts of the green leaf tissue were prepared as described for the cured samples, by immersing the tissue in boiling water followed by autoclaving.

Extracts were assayed for free and bound *cis*- and *trans*-*o*-HCA by a fluorometric procedure (5) in which the Turner Model 110 Fluorometer was used.[3] This procedure provides readings for free *trans*-*o*-HCA, total free *o*-HCA, total *trans*-*o*-HCA, and total *o*-HCA. Values for free *cis*-*o*-HCA and bound *cis*- and *trans*-*o*-HCA are calculated by appropriate subtractions (total free-free trans = free *cis*; total trans-free *trans* = bound *trans*; total *o*-HCA-total trans = total *cis*; total *cis*-free *cis*-bound *cis*).

Results and Discussion

The *o*-HCA in all samples of cured deer's tongue tissue occurred primarily as the free *cis* isomer (Table I). Mean contents of this form amounted to 2 to 3% of the dry weight of the tissue. Mean levels of bound *cis*-o-HCA were less than 20% as high as levels of the free form. Contents of the trans isomer averaged about 30 to 40% as high as *cis* contents; generally the trans isomer was about equally divided between the free and bound forms. Levels of *cis*- and *trans*-*o*-HCA in leaf midribs were somewhat lower than in leaf

[3]Mention of specific products is for identification only and does not imply endorsement by the United States Department of Agriculture.

blades, and *o*-HCA levels in the single sample of seeds were much lower than in cured leaves.

In some respects it would be preferable to speak of "coumarin" rather than "free *cis*-*o*-HCA," for the lactone rather than the free acid occurred in the cured samples. Results are expressed in terms of *o*-HCA in this paper to facilitate comparison among the various forms. Levels of coumarin can be calculated readily by multiplying the free *cis*-*o*-HCA values by 0.890, the ratio of the molecular weight of coumarin to that of *o*-HCA.

TABLE I

O-HYDROXYCINNAMIC ACID IN CURED DEER'S TONGUE TISSUE

Sample description	Form of *o*-HCA		*o*-HCA Percentage* (Mean±SE)
Whole leaves	*cis*		
		free	2.68 ± 0.15
		bound	0.49 ± 0.34
	trans		
		free	0.41 ± 0.13
		bound	0.86 ± 0.15
	Total		4.44 ± 0.35
Leaf midribs	*cis*		
		free	2.02 ± 0.26
		bound	0.02 ± 0.02
	trans		
		free	0.26 ± 0.06
		bound	0.29 ± 0.07
	Total		2.59 ± 0.31
Leaf blades	*cis*		
		free	2.64 ± 0.28
		bound	0.23 ± 0.06
	trans		
		free	0.60 ± 0.08
		bound	0.57 ± 0.17
	Total		4.04 ± 0.35
Pulverized tissue	*cis*		
		free	2.61 ± 0.02
		bound	0.07 ± 0.02
	trans		
		free	0.49 ± 0.01
		bound	0.37 ± 0.01
	Total		3.54 ± 0.03
Seed †	*cis*		
		free	0.32
		bound	0.05
	trans		
		free	0.07
		bound	0.23
	Total		0.67

* Dry weight basis, mean of 5 samples.
† One sample only.

The mean total *o*-HCA content of the three sampled leaves from chamber-grown plants was 12.7% (dry weight basis). Approximately 88% of the compound was present as the *trans* isomer, and over 99% was in the bound form. It is apparent that these fresh leaves differed drastically from the cured leaves in level of *o*-HCA as well as in free: bound and *trans*:*cis* ratios. The total *o*-HCA level was about twice as high as levels ordinarily observed in sweetclover, but free:bound and *trans*:*cis* ratios were similar to those encountered in young chamber-grown sweetclover leaves (2).

Fresh leaves harvested approximately 2 ½ months after plants had been moved from the growth chamber to the greenhouse were still very high in *o*-HCA content, and little of the compound was present in the free form (Table II). Midribs of the longest leaves were lower in o-HCA than the surrounding leaf blade tissue. In blades of the longest leaves, the *cis* isomer predominated (about 77% of the total), but in young leaves the *trans* isomer was predominant (about 73% of the total). The relatively high *trans*:*cis* ratio in chamber-grown and young greenhouse-grown leaves, and the low ratio in older greenhouse-grown leaves and cured tissue suggest that in deer's tongue, as in sweetclover (6), light of suitable quality and duration is required for the conversion of bound *trans*-*o*-HCA to the corresponding *cis* isomer.

Paper chromatography and fluorescence spectra were used to determine whether the

TABLE II

O-HYDROXYCINNAMIC ACID IN FRESH LEAVES FROM GREENHOUSE-GROWN DEER'S TONGUE PLANTS

Sample description	Form of *o*-HCA		*o*-HCA Percentage* (Mean±SE)
Young leaves	*cis*		
		free	0.06 ± 0.024
		bound	3.14 ± 0.33
	trans		
		free	0.01 ± 0.006
		bound	8.78 ± 0.38
	Total		11.99 ± 0.38
Longest leaves Midribs	*cis*		
		free	0.05 ± 0.010
		bound	2.60 ± 0.47
	trans		
		free	0.01 ± 0.003
		bound	1.77 ± 0.30
	Total		4.43 ± 0.49
Remainder of leaf tissue	*cis*		
		free	0.21 ± 0.108
		bound	7.35 ± 0.46
	trans		
		free	0.002 ± 0.002
		bound	2.28 ± 0.33
	Total		9.84 ± 0.71

* Dry weight basis, mean of 9 plants.

assay procedure provided a reliable measure of *o*-HCA in deer's tongue extracts. The chromatographic comparisons involved an extract of chamber-grown leaves (in which, as previously noted, *o*-HCA occurred principally as the bound trans isomer) chromatographed on Whatman No. 1 filter paper with the following solvents: methanol; 2% acetic acid; *n*-propyl alcohol, glacial acetic acid, and water, 8:1:2, v/v/v; and *n*-propyl alcohol, concentrated ammonium hydroxide, and water, 8:1:2, v/v/v. Synthetic *trans-o*-HCA glucoside (kindly supplied by Dr. T. Kosuge, University of California, Davis) was used as a standard. The synthetic compound appeared as a strong absorbing spot on chromatograms viewed under 260 nm ultraviolet light. In every solvent system a pronounced 260 nm-absorbing spot was observed in the chromatographed plant extract, at an R_f corresponding very closely to that of the standard. The Aminco-Bowman Spectrophotofluorometer was used for comparison of fluorescence spectra. As shown in Figure 1, there were no readily apparent differences between the fluorescence spectrum of a hydrolyzed extract of chamber-grown deer's tongue leaves and that of authentic *trans-o*-HCA. On the basis of this comparison and the chromatographic results, it is reasonable to conclude that the readings made with the Turner Fluorometer provided an accurate indication of *o*-HCA levels.

The predominance of bound *cis-o*-HCA in older leaves of greenhouse-grown plants and free *cis*-o-HCA (*i.e.*, coumarin) in cured samples suggests that the bound form can be hydrolyzed by an endogenous enzyme to yield free coumarin. Preliminary tests of

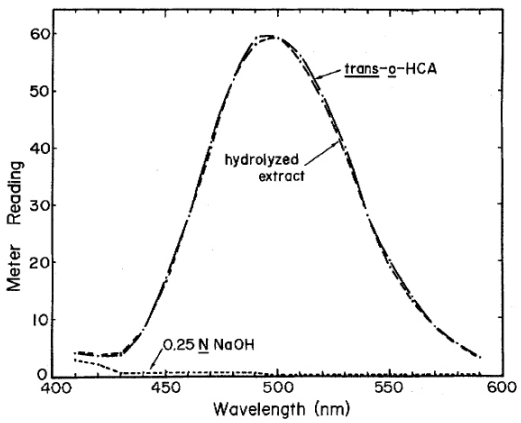

FIG. 1. Fluorescence spectra of *trans-o*-hydroxycinnamic acid and hydrolyzed extract of chamber-grown deer's tongue leaves. Concentration of *trans-o*-HCA: 5.0×10^{-4} micromoles/ml. The hydrolyzed extract was diluted to an equivalent concentration based on fluorescence as read with the Turner Fluorometer. Solvent: 0.25 *N* NaOH. Excitation wavelength: 360 nm.

homogenates derived from greenhouse-grown plants revealed that deer's tongue, like sweet clover (8), indeed contains a *β*-glucosidase which hydrolyzes *cis*-*o*-HCA glucoside but is much less active against *trans*-*o*-HCA glucoside. The virtual absence of free *o*-HCA in hot water extracts of fresh leaf samples indicates that this *β*-glucosidase is not active in the healthy, intact tissue. However, during the tissue disruption associated with curing, the enzyme apparently is highly effective in hydrolyzing bound *o*-HCA.

It appears that the deer's tongue constituent most desired by the tobacco industry is coumarin (1, 9). If this is indeed the case, it would seem desirable to ascertain whether a relationship exists between growth stage and *cis*-*o*-HCA glucoside content in field-grown plants, and to devise a curing procedure effecting the maximal production and preservation of free coumarin.

Literature Cited

1. Anonymous. 1969. Coumarin—not good for rats but man uses it differently. *Tobacco Reporter* 96(6): 69, 71.
2. Gorz, H. J. & F. A. Haskins. 1964. Occurrence of *o*-hydroxycinnamic acid in species of *Melilotus* and *Trigonella*. *Crop Science* 4: 193-196.
3. Haskins, F. A. & H. J. Gorz. 1961. A reappraisal of the relationship between free and bound coumarin in *Melilotus*. *Crop Science* 1: 320-323.
4. Haskins, F. A. & H. J. Gorz. 1963. Glucosides of coumarinic and *o*-coumaric acids in the tonka bean. *Science* 139: 496-497.
5. Haskins, F. A. & H. J. Gorz. 1970. Fluorometric assay of free and bound, *cis*- and *trans*-*o*-hydroxycinnamic acid in a single plant extract. *Crop Science* 10: 608-609.
6. Haskins, F. A., L. G. Williams & H. J. Gorz. 1964. Light-induced trans to *cis* conversion of *β*-D-glucosyl *o*-hydroxycinnamic acid in *Melilotus alba* leaves. *Plant Physiol.* 39: 777-781.
7. Kosuge, T. 1961. Studies on the identity of bound coumarin in sweet clover. *Arch. Biochem. Biophys.* 95: 211-218.
8. Kosuge, T. & E. E. Conn. 1961. The metabolism of aromatic compounds in higher plants. III. The *β* -glucosides of *o*-coumaric, coumarinic, and melilotic acids. *J. Biol. Chem.* 236: 1,617-1,621.
9. Krochmal, A. 1969. Deer's tongue, *Trilisa odoratissima*, a useful plant of southeastern United States. *Econ. Botany* 23: 185-186.